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Chronic exogenous kisspeptin administration accelerates gonadal development in basses of the genus *Morone*

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ABSTRACT

The present study assesses the effects of chronic administration of peptides to fish, termed kisspeptins, which are the products of the KISS1 and KISS2 genes, and have been shown to control the development of puberty in animals. Using ecologically and commercially important species (white bass, *Morone chrysops*, striped bass, *Morone saxatilis*, and their hybrid) as comparative models, we determined that repeated bi-weekly injections (over 7 weeks) differentially accelerate puberty, as evidenced by increases in the prevalence of spermatozoa in the testes of juvenile fish. Moreover, in sexually mature fish, kisspeptin treatment led to increased gonad weight, gonadosomatic index, and spermatocrit in some white and striped bass. Additionally, mature white bass treated with kisspeptins showed an advancement in oocyte development as determined by histological examination. These gonadal changes occurred in the absence of any photothermal manipulation or hormone injections. To date, this is the first description of kisspeptin-mediated pubertal initiation in fish, and the first evidence that kisspeptins could modulate gonad maturation. Although it remains to be determined how kisspeptins may best be utilized in practice, our findings are a basis for future studies to characterize the molecular underpinnings of the KISS system in various fish species.

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1. Introduction

The KISS1 gene was first discovered in the context of cancer, specifically melanoma, where it was demonstrated to be a suppressor of metastasis (Lee et al., 1996; Lee and Welch, 1997a, 1997b; Nash and Welch, 2006; Nash et al., 2007; Beck and Welch, 2010; McNally et al., 2010). Since that time, the KISS1 gene and the KISS1 receptor (KISS1R or GPR54) have emerged as the principal system mediating pubertal development in vertebrate animals. This perspective is supported by studies demonstrating that both KISS1R-knockout mice and mutations in KISS1R in patients cause reproductive/pubertal failure, notably autosomal recessive idiopathic hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003; Mitani et al., 2010). The products of the KISS1 gene are termed kisspeptins, which bind to KISS1R (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The full-length KISS1 protein is 54 amino acids and is termed KP54 (alternatively named metastin) which can be proteolytically cleaved into shorter fragments (e.g., KP10, KP13, and KP14) representing the C-terminus of KP54, and which signal through KISS1R with presumably equal activity (Kotani et al., 2001). Exogenous kisspeptins have been administered to numerous vertebrate animals including humans and rodents, and have been shown to stimulate gonadotropin release including luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (Gottsch et al., 2004; Dhillo et al., 2005, 2007; Cho et al., 2009; Beck and Welch, 2010; George et al., 2011).

More recently, the KISS1 gene and a paralog termed KISS2 have been identified in teleost fishes (Filby et al., 2008; Kanda et al., 2008; van Aerle et al., 2008; Felip et al., 2009; Kitahashi et al., 2009; Li et al., 2009; Mitani et al., 2010; Shi et al., 2010; Yang et al., 2010; Servili et al., 2011). According to synteny analysis, the KISS gene was duplicated before the divergence of sarcopterygians and actinopterygians, but it seems that the KISS2 gene was lost in placental mammals (Zohar et al., 2010). KISS1 and KISS2 gene sequences are dissimilar; however, they have some sequence similarity at the amino acid level (60–80%) of the smallest known kisspeptin, the decapeptide KP-10 (Mitani et al., 2010). This disparity in amino acid sequences could result in different efficacies on the KISS receptor(s) (Zohar et al., 2010). Indeed, this notion is supported by a study on transfected cell lines expressing either of the kisspeptin-receptors identified in fish (GPR54-1 and GPR54-2) where KISS1 exhibited higher sensitivity to GPR54-1, while GPR54-2 responded to KISS1 and KISS2 with similar sensitivity (Lee et al., 2009). Studies examining the in vivo effects of kisspeptin administration in fish have primarily focused on the hormonal (mainly luteinizing hormone (LH) and follicle-stimulating hormone (FSH) profiles of fish at timepoints soon (e.g., hours) after exogenous administration, or have involved the temporal and spatial characterization of KISS or KISS

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receptor mRNA expression during seasonal variation. More specifically, in goldfish *Carassius auratus*, intraperitoneal administration of KISS1 resulted in an increase in serum LH, while KISS2 treatment showed little effect (Li et al., 2009). Alternatively, in European sea bass, *Dicentrarchus labrax*, intramuscular injections of KISS2 exerted superior effects in terms of LH secretion over KISS1 (Felip et al., 2009). In orange-spotted grouper, *Epinephelus coioides*, intraperitoneal injection of KISS2 decapeptide significantly increased gnrh1 transcript levels in the hypothalamus and follicle-stimulating hormone beta (fshb) transcript levels in the pituitary at 6 and 12 h post-injection (Shi et al., 2010).

Although considerable progress has been made, the precise neuroendocrine mechanisms underpinning reproductive success and failure have remained largely hidden, frustrating efforts to improve fish breeding practices (Zohar et al., 2010). The recent discovery of the KISS system as a central regulator of GnRH and integrator of environmental cues reinforces the fact that our knowledge of the molecular actors and their roles in puberty and fertility is still deficient (Zohar et al., 2010). For our purposes, exploiting the KISS/kisspeptin system, in the setting of aquaculture, abounds with theoretical promise. Maintaining stocks of fish until sexual maturation can be an economic burden to commercial producers due to feed and space considerations, as well as the increased risk of stress and/or disease-related losses (Taranger et al., 2010). This is of particular importance in species where onset of puberty takes substantial time such as groupers (*Serranidae*), tunas (*Scombridae*), and sturgeons (*Acipenseridae*) (Taranger et al., 2010). It is also important in interspecific hybrids, where maintenance of pure-line broodstock of more than one species is necessary to produce the F1 intercross progeny, such as hybrid catfish (*Ictalurus punctatus* × *Ictalurus furcatus*), hybrid tilapia (*Oreochromis* spp.), and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Thus, accelerating puberty for earlier reproduction, exerting more control over reproduction to extend breeding seasons, or better synchronizing the timing of spawning to overlap between different species for hybrid production, are all scenarios that could improve the cost-efficiency of many industries (Taranger et al., 2010). Furthermore, propagation of endangered or imperiled species may benefit from improvements in reproductive efficiencies and outputs.

However, aside from such commercial application, understanding the role of kisspeptins and their associated receptors at a fundamental level is equally important in the quest to better understand and exert more control over fish reproduction. To date, no study has demonstrated that delivery of exogenous kisspeptins (either KISS1 or KISS2) could affect gonadal condition or quality. Previous studies have instead focused on either identifying the presence of a KISS1/KISS2/KISS1R axis in different fishes or by characterizing the early molecular signaling events after administration. In the present study, we sought to characterize the physiological consequences of repeated exogenous kisspeptin administration to sexually immature and sexually mature fish with a specific emphasis on documenting the size and histological development level of the gonad. Here, using two model species of closely related fish within the family *Moronidae*, and their hybrid, we demonstrate that KISS1 and KISS2 decapeptides can differentially accelerate puberty onset, increase gonad mass, oocyte size, and sperm density within milt.

2. Materials and methods

2.1. Fish

The effects of kisspeptin treatment on two different age groups of mixed sex white bass *M. chrysops* that were 8 months (juvenile) and 19 months of age (sexually mature); one 8 month group of juvenile mixed sex striped bass, *M. saxatilis*, one 5 ½ year group of female striped bass (sexually mature), and one 8 month group of hybrid striped bass (juvenile) (Table 1). Fish were either bred on-site at the Stuttgart National Aquaculture Research Laboratory in Stuttgart, AR, USA or obtained from the North Carolina State Pamlico Field Lab, Aurora, NC, USA. Fish were fed a commercial diet daily to satiation. Fish were maintained in common garden fashion; to monitor each individual fish throughout the course of the study, all fish were tagged with alcohol sterilized PIT tags (Biomark, Boise, ID, USA; measuring 8.5 mm × 2.12 mm and 0.067 g) using a standard 12 gauge tagging syringe in the dorsal musculature (DM). Before PIT tagging, and before each peptide treatment, fish were anesthetized in well water containing clove oil (Sigma-Aldrich, St. Louis, MO, USA) to initial loss of equilibrium.

2.2. Water quality

Fish were randomly distributed by species (approximately 30 fish per tank, except for adult striped bass which were stocked at a density of six fish per tank) among three 600 L tanks with forced aeration and flow-through well water at 21.8 ± 1.1 °C, pH 7.8, and dissolved oxygen of 8.0 mg/L. No manipulations were made in water temperature or photoperiod.

2.3. Peptides

Peptides were designed based on the previously reported (Felip et al., 2009) amino acid sequences of the kisspeptin-10 region in the European sea bass (*D. labrax*); a closely related species with a high homology in GnRH to fish of the genus *Morone* (Zmora et al., 2002). Two amidated peptides termed KISS1 (YNLNSFGLRY-NH2) and KISS2 (FNFNPFGLRF-NH2) were synthesized by CPC Scientific (Sunnyvale, CA, USA). Peptides were diluted in Dulbecco's phosphate-buffered saline (PBS; Cellgro by Mediatech, Manassas, VA, USA) and were injected in the DM twice weekly at a dose of 250 ng/g body weight for 8 weeks ranging from January to May 2010 (Table 1). Control fish were injected with PBS alone. Dosing was selected based on previously reported dosages that elicit gonadotropin release in European sea bass (Felip et al., 2009). Doses were adjusted to account for individual weight gain during the study by weighing fish at the beginning of the study, on week 4, and near the termination of the study (week 7). There

Table 1
Species and characteristics of fish used to examine gonad-level responses to chronic exogenous kisspeptin administration.

Species	Age at start (months) and classification	Starting weight (mean ± SEM g)	Ending weight (mean ± SEM g)	Number of individuals	Number of injections
White bass *Morone chrysops*	19 Mature	289.8 ± 7.7	302.1 ± 8.9	43 (22 male; 21 female)	15
White bass *M. chrysops*	8 Juvenile	79.6 ± 1.6	126.7 ± 2.8	90 (46 male; 44 female)	14
Striped bass *M. saxatilis*	68 Mature	1771.6 ± 52.9	2116.2 ± 67.7	18 (all female)	14
Striped bass *M. saxatilis*	8 Juvenile	111.7 ± 1.8	207.7 ± 3.8	88 (51 male; 37 female)	14
Hybrid striped bass *M. chrysops* × *M. saxatilis*	8 Juvenile	134.5 ± 3.2	208.7 ± 5.1	80 (32 male; 48 female)	15

were no mortalities associated with treatment and no untoward side effects observed.

2.4. Milt volume and spermatocrit

At the conclusion of the study, freshly stripped milt (from at least 27 males per treatment) was collected in a 50 mL polypropylene conical tube (BD Biosciences, Franklin Lakes, NJ). Spermatocrit, a measure of the proportion of milt occupied by spermatozoa, was determined using milt collected with non-heparinized microhematocrit tubes (75 mm in length, 1.2 mm internal diameter) to quantitatively assess differences in spermatozoan density relative to milt volume. Milt was drawn into microhematocrit tubes until 60–80% of the tube volume was occupied by semen. A single end of the tube was then sealed with clay, and the tubes were centrifuged at 5500 *g* for 15 min. Two samples were collected from each individual male, and at least 19 males were sampled for each treatment. All spermatocrits were determined within 1 h of collection.

2.5. Histology sampling and processing

Fish were necropsied after seven weeks and gonads were immediately fixed in 10% neutral buffered formalin for 48 h. Fixed tissues were rinsed with water and stored in 50% isopropanol. Stored tissues were dehydrated in isopropanol, cleared in Citri-Solv (Fisher Scientific, Pittsburgh, PA, USA) and embedded in Paraplast Plus (Oxford Labware, St. Louis, MO, USA). Tissues were sectioned (4–6-μm thick) and stained with hematoxylin and eosin (Stevens and Wilson, 1996).

2.6. Histological examination of gonadal tissues

All tissues were scored in blinded fashion. Testes were scored for the presence of spermatozoa using the cellular stages identified by Groman (1982). In female fish, ovaries were assessed for oocytic development as described by Groman (1982) and Berlinsky et al. (1995); briefly, in four 40× microscopic fields, the following were counted: later stage oocytes at stage VI and oocytes at earlier stages of development, i.e., oocytes ≤ stage V (Groman, 1982). In the four microscopic fields, the percentage of mature oocytes was calculated relative to the total number of oocytes (number of ova at stage VI ÷ number of ova at stages I–VI × 100). As described by Groman (1982), the stage characteristics are as follows: Stage I ova are undifferentiated oogonia that occur in nests and contain lightly acidophilic-staining cytoplasm with large rounded central nuclei each containing a prominent nucleus; stage II ova are larger and stain basophilically; each contains a disproportionately large central nucleus with diffuse chromatin; stage III ova are larger oocytes that have a dramatically increased basophilic ovaplasm in comparison to stage II ova, and numerous provitelline nucleoli appear in the karyoplasm as the true nucleolus migrates to a more peripheral position; in stage IV ova, the oviplasm begins to stain basophilically, euvitelline nucleoli appear along the nuclear membrane, the nucleus stains acidophilically, and no true nucleolus is evident and an increase in yolk granules and fat vacuoles occurs in the oviplasm and a distinct zona radiata can be distinguished in the follicular epithelium for the first time; in stage V ova, there is a tremendous increase in yolk vesicles, except in the basophilic blue-gray oviplasm directly adjacent to the acidophilic nucleus and a thin blue-gray oviplasm is located beneath the zona radiata and fewer euvitelline nucleoli are present in the acidophilic nucleus; and finally, stage VI ova contain oviplasm filled with larger fat vacuoles and acidophilic yolk

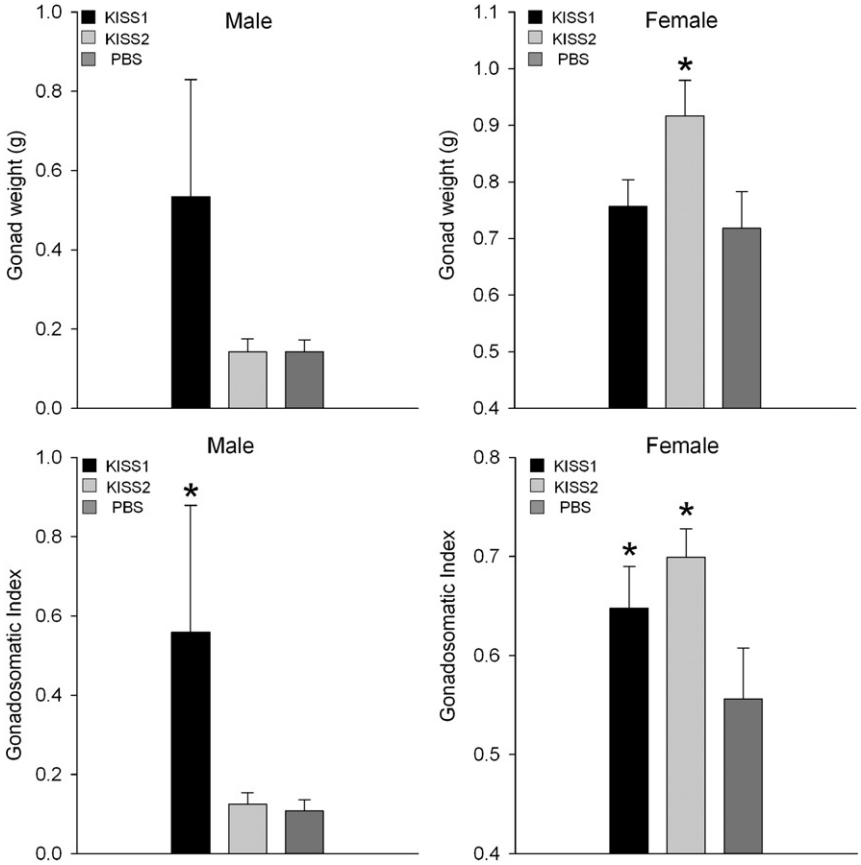

Fig. 1. Gonad weight and gonadosomatic index (GSI) of male and female juvenile white bass *Morone chrysops* after 14 bi-weekly injections of 250 ng/g body weight of KISS1 or KISS2 decapeptides or diluent phosphate-buffered saline (PBS). Asterisk indicates a significant difference from PBS injected fish. The number of animals examined was as follows: male fish; KISS1 (n = 11), KISS2 (n = 16), PBS (n = 14); female fish; KISS1 (n = 18), KISS2 (n = 12), PBS (n = 14).

granules, except for a thin layer of homogenous blue-gray cytoplasm adjacent to the zona radiata. Sections were viewed and photographed at 50× magnification with a Zeiss Axiovert 40 microscope fitted with a Zeiss Icm1 Axiocam, and oocyte diameters were measured with Zeiss AxioVision software (Zeiss, Thornwood, New York, USA). Oocyte diameters were calculated by measuring the follicle diameters from 27 to 35 stage VI oocytes from no fewer than 5 female fish from each treatment.

2.7. Statistics

Statistical differences were evaluated using either JMP version 9 (SAS Institute, Inc., Cary, NC, USA) or SigmaPlot 11 (Systat Software, San Jose, CA). Differences between the mean gonad size, GSI, oocyte diameter, and spermatocrit for all treatments were evaluated using a one-way ANOVA. Normality was tested using a Shapiro–Wilk test; if normality failed or unequal variances were discovered, a Mann–Whitney–Wilcoxon Rank Sum test was performed. Differences in prevalence of spermatozoa in histological analysis of testes were calculated using a Fisher's exact test. Differences were considered significant if $P \leq 0.05$.

3. Results

3.1. Weight gain

In juvenile white bass, striped bass, and hybrid striped bass, there were no differences in weight gain by treatment group over the course of the study. There were no differences in weight gain in sexually mature white bass by treatment group. In adult striped bass females, the KISS1-treated fish, but not the KISS2-treated fish, gained significantly more weight ($P = 0.016$) than PBS-treated fish. Weight gains in adult female striped bass by treatment group were (% increase ± SEM) KISS1: 19.2 ± 1.34, KISS2: 16.3 ± 1.4, and PBS: 12.5 ± 1.9.

3.2. Gonad weight and gonadosomatic index

Gonad size and GSI are reported in mean ± SEM. In juvenile white bass males, the total weight of testes was not significantly different in KISS1-treated (0.54 ± 0.29 g) or KISS2-treated fish (0.14 ± 0.03) as compared to PBS-treated fish (0.14 ± 0.03 g) (Fig. 1). Of KISS1-treated juvenile white bass males, 72.7% of fish had testes of greater mass than the average mass of PBS-treated fish, while 43.8% of KISS2-treated juvenile white bass males had testes of greater mass than the average mass of PBS-treated fish. In these same treatment groups of fish, the GSI of KISS1-treated males (0.56 ± 0.32%), but not KISS2-treated males (0.12 ± 0.03), was significantly greater than PBS-treated males (0.11 ± 0.03) ($P = 0.05$) (Fig. 1). In juvenile female white bass, only the administration of KISS2 (0.92 ± 0.06 g) resulted in an increase in ovary weight over PBS-treated fish (0.71 ± 0.06) ($P = 0.03$) (Fig. 1). However, both KISS1 (0.65 ± 0.04; $P = 0.03$) and KISS2 (0.70 ± 0.03; $P = 0.01$) treatments resulted in a significant increase in GSI as compared to PBS-treated fish (0.56 ± 0.05). In eight month old juvenile striped bass, there were no significant differences in gonad size (either testis or ovary) or GSI in any treatment (Fig. 2). In hybrid striped bass juveniles, there were no differences in gonad weight or GSI in any treatment (not shown).

In adult male white bass, the testes of both KISS1 (12.4 ± 1.7 g; $P = 0.03$) and KISS2 (11.7 ± 1.2; $P = 0.03$) treated fish were significantly larger than PBS (8.8 ± 0.9) treated fish (Fig. 3). KISS1- and KISS2-treated mature female white bass had ovary weights of 32.8 ± 4.5 g and 37.5 ± 5.8 g respectively, but were not significantly different

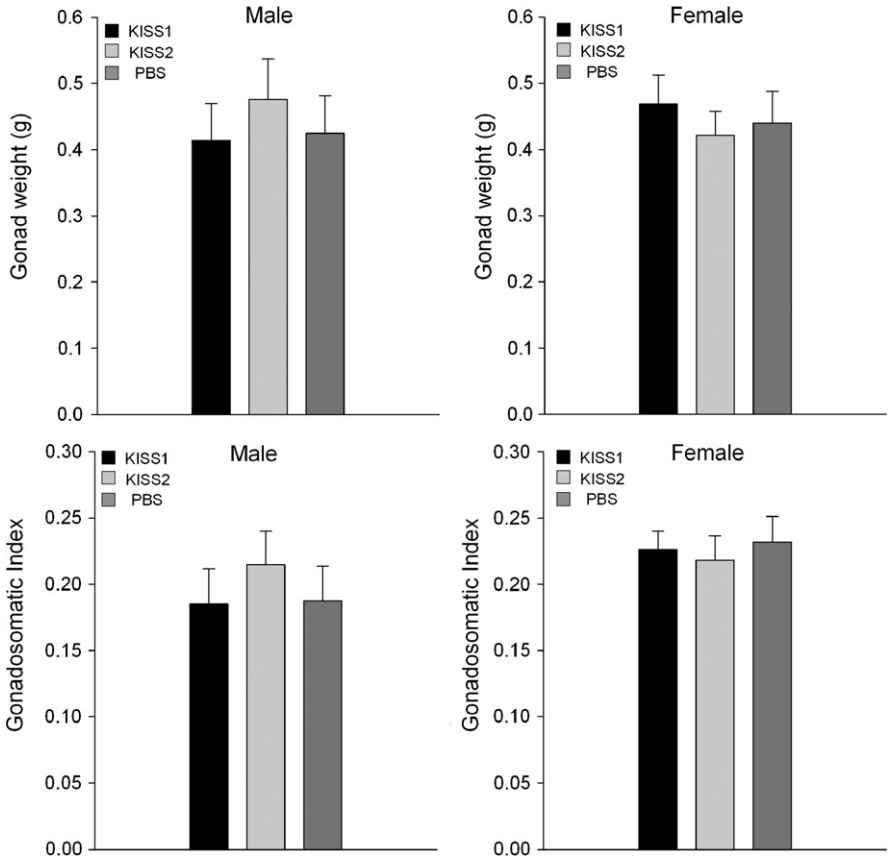

Fig. 2. Gonad weight and gonadosomatic index (GSI) of male and female juvenile striped bass *Morone saxatilis* after 14 bi-weekly injections of 250 ng/g body weight of KISS1 or KISS2 decapeptides or diluent phosphate-buffered saline (PBS). The number of animals examined was as follows: male fish; KISS1 (n = 13), KISS2 (n = 12), PBS (n = 12); female fish; KISS1 (n = 17), KISS2 (n = 17), PBS (n = 17).

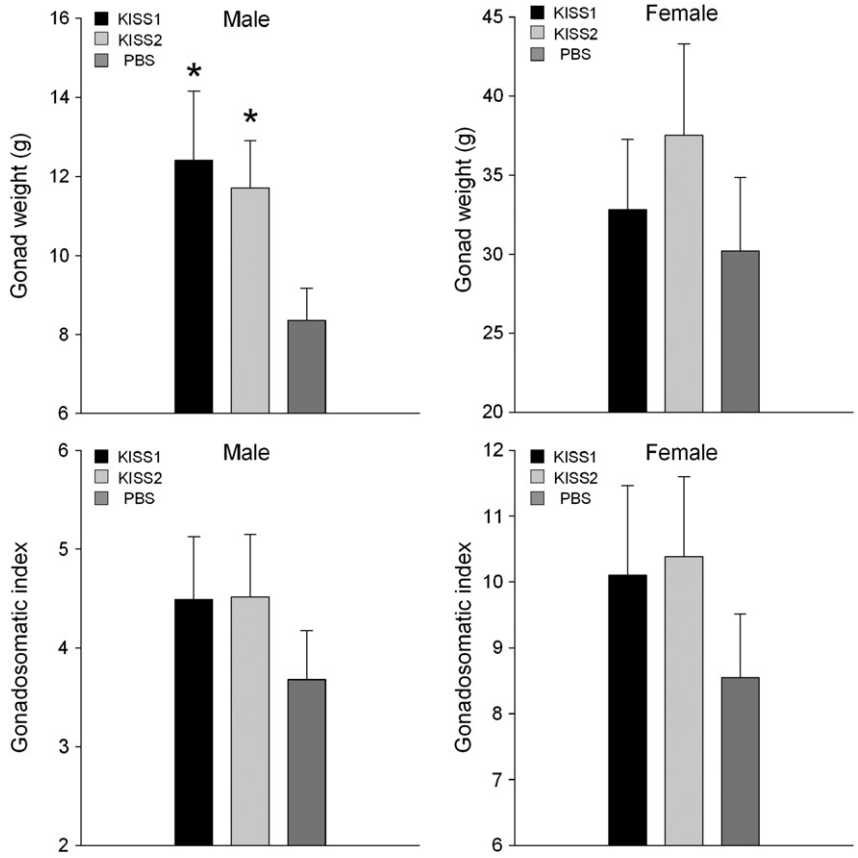

Fig. 3. Gonad weight and gonadosomatic index (GSI) of sexually mature male and female white bass *Morone chrysops* after 15 bi-weekly injections of 250 ng/g body weight of KISS1 or KISS2 decapeptides or diluent phosphate-buffered saline (PBS). Asterisk indicates a significant difference from PBS injected fish. The number of animals examined was as follows: male fish; KISS1 (n = 6), KISS2 (n = 8), PBS (n = 9); female fish; KISS1 (n = 12), KISS2 (n = 9), PBS (n = 5).

from PBS-treated females (30.2 ± 4.6) (Fig. 3). In both males and females, KISS1 (male GSI, 4.5 ± 0.63%; female GSI, 10.1 ± 1.4) and KISS2 (male GSI, 4.5 ± 0.63; female GSI, 10.4 ± 1.23) treated fish were not significantly different (P > 0.05) in GSI than PBS (male GSI, 3.6 ± 0.49; female GSI, 8.5 ± 0.97) treated fish. In adult female striped bass, only the GSI of KISS2-treated fish were significantly greater (P = 0.03) than the PBS control (Fig. 4).

3.3. Histology, milt volume, and spermatocrit

3.3.1. Juvenile fish

In 8 month old white bass, 80% of testes examined within the KISS1 treated group contained nests of spermatozoa, which was significantly greater (P = 0.003) than PBS-treated fish where only 15.3% of fish exhibited spermatozoa (Fig. 5). In KISS2-treated fish, 38% exhibited spermatozoa within testes, which was not significantly different from PBS. Interestingly, while not intentionally manually expressed, 20% of KISS1-treated 8 month old white bass were spermiating when routinely handled for necropsy. No other treatments in this age group of white bass showed outward signs of spermiation. There were no histological differences in female 8 month old white bass where all oocytes examined were predominately in stages I through III in development consisting primarily of perinucleolar and lipid droplet oocytes. In 8-month old striped bass, there were no histological differences in the testes with spermatozoa detected in 5.9% of KISS1-treated fish, 23.1% of KISS2-treated fish, and 18.7% of PBS-treated fish. In 8 month old striped bass females, there were no differences in oocyte development where all oocytes examined were in stages I through III of development. In hybrid striped bass juveniles, there were no differences in gonad structure or stage among any treatment (data not shown).

3.3.2. Adult fish

In 19 month old male white bass, irrespective of treatment, all testes examined histologically contained an abundance of spermatozoa and were not qualitatively different. Milt volumes per kg of body weight reported in mean ± SEM were: KISS1-treated (4.15 ± 0.34 mL/kg), KISS2-treated (4.35 ± 0.37 mL/kg), and PBS-treated (4.82 ± 0.42 mL/kg), which were not significantly different from each other. Spermatocrit values were KISS1-treated (78.5 ± 1.6%), KISS2-treated (77.8 ± 1.9%), and PBS-treated (73.5 ± 1.3%). However, only KISS1-treated fish had spermatocrit levels that were statistically greater than PBS-treated fish (KISS1, P = 0.022) (KISS2, P = 0.062). In female fish injected with KISS1 and KISS2, 54.8 ± 6.3% and 58.6 ± 2.4% of oocytes were in stage VI of development, while PBS-treated fish ovaries showed 41.8 ± 6.1% of oocytes in stage VI (KISS1 versus PBS, P = 0.18; KISS2 versus PBS, P = 0.050; KISS1 and KISS2 combined versus PBS, P = 0.058) (Fig. 6). The mean diameter of oocytes within ovaries of females was as follows (mean diameter ± SEM): KISS1 = 589.3 ± 9.5 μm, KISS2 = 578.7 ± 7.1 μm, and PBS = 542.2 ± 16.1 μm. Oocyte diameters from both KISS1- and KISS2-treated were significantly larger (P = 0.016 and P = 0.031 respectively) than PBS-treated females. In adult striped bass females, there were no microscopic differences in oocytic stage among all treatments, with oocytes being predominately stages III and IV (not shown).

4. Discussion

The concept of exploiting kisspeptins to modulate aspects of reproduction is not new, as evidenced by studies employing rodent models and clinical trials in human patients (Seminara et al., 2003; Gottsch et al., 2004; Dhillo et al., 2005, 2007; George et al., 2011). While a host of preliminary studies in fish have indicated basic conservation of function with mammals (Tena-Sempere et al., 2012)

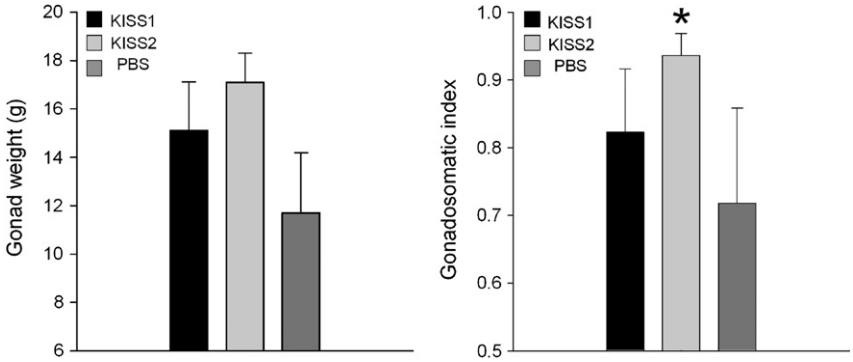

Fig. 4. Gonad weight (*left panel*) and gonadosomatic index (GSI; *right panel*) of sexually mature female striped bass *Morone saxatilis* after 14 bi-weekly injections of 250 ng/g body weight of KISS1 or KISS2 decapeptides or diluent phosphate-buffered saline (PBS). Asterisk indicates a significant difference from PBS injected fish. The number of animals examined was as follows: KISS1 (n = 6), KISS2 (n = 6), PBS (n = 6).

little is known regarding how these genes/gene products control puberty in fish. Indeed, puberty, a critical event in the life history of fish, is of major importance, both from a basic and applied science perspective and must be completely understood before it can be mastered (Zohar et al., 2010). The time at which fish reach sexual maturity remains an important parameter in numerous fisheries-related disciplines including commercial foodfish operations, ornamental fish propagation, and fisheries population management/sportfish programs (Holland et al., 2000). Irrespective of fish species, one of the major limiting factors to furthering the development and intensification of these respective disciplines lies in reproductive inefficiencies, such as the prolonged time to reach sexual maturation and poor fecundity, which produce barriers in the supply of fish. Such barriers are also prevalent in the propagation of our selected model species employed here; the striped bass, white bass, and their hybrid, which collectively can be viewed as species of ecological, recreational, and commercial importance (Garber and Sullivan, 2006). Accordingly, in the present study, using exogenous kisspeptin treatments, we sought to drive gross and histologic phenotypic changes in the gonad in the absence of any temperature conditioning (e.g., cold-banking), photoperiod manipulation, and without the usage of injection of other hormone preparations (e.g., hCG).

To date, most of the evidence linking kisspeptins to puberty in fish is circumstantial in nature, as coming from gene expression analyses (Tena-Sempere et al., 2012). Here, the increased prevalence of spermatozoa in the testes of juvenile male white bass treated with KISS1 may be the first description of kisspeptin-mediated pubertal initiation in teleost fish. Curiously, in both juvenile striped bass and hybrid striped bass treated with KISS decapeptides, the fish were unresponsive in terms of gonad mass/GSI and histologic changes,

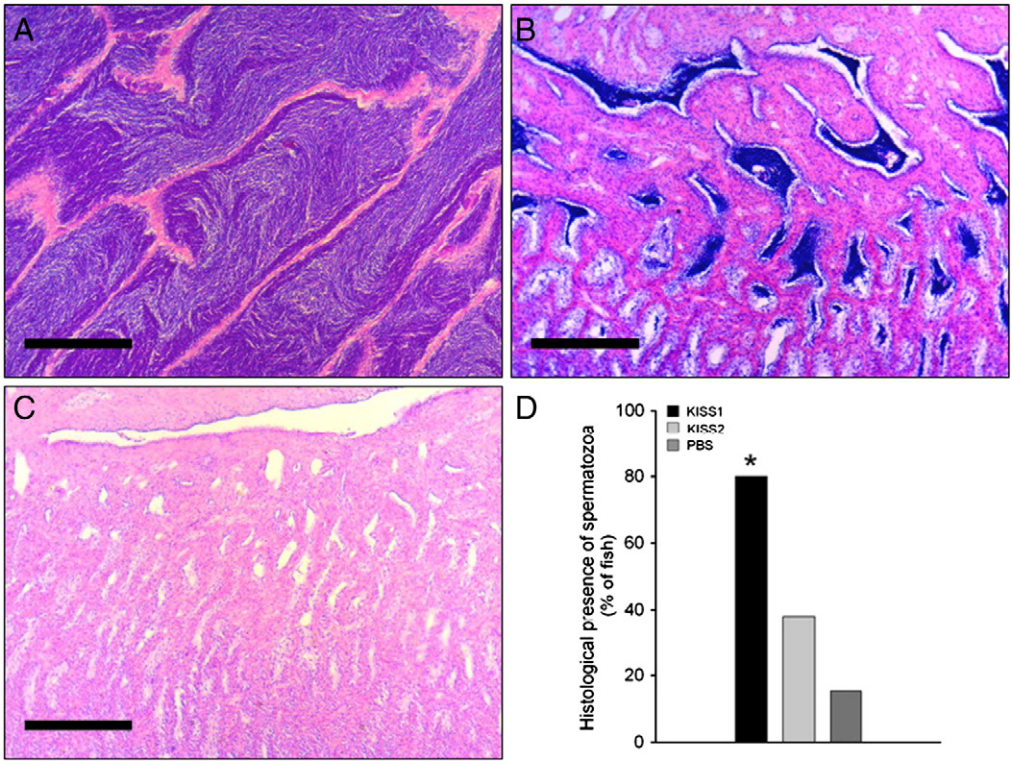

Fig. 5. Representative photomicrographs demonstrating testicular development of 8 month old male white bass *Morone chrysops*. A.) KISS1 treated fish testis showing distinct spermatozoa-filled ducts. B.) KISS2 treated fish with ducts containing spermatozoa. C.) Phosphate-buffered saline (PBS) treated fish showing ducts void of spermatozoa. (Scale bar = 500 μm). D.) Percentage of fish by treatment containing histological evidence of spermatozoa within testes. Asterisk indicates a significant difference from PBS injected fish. The number of animals examined was as follows: KISS1 (n = 10), KISS2 (n = 16), PBS (n = 13).

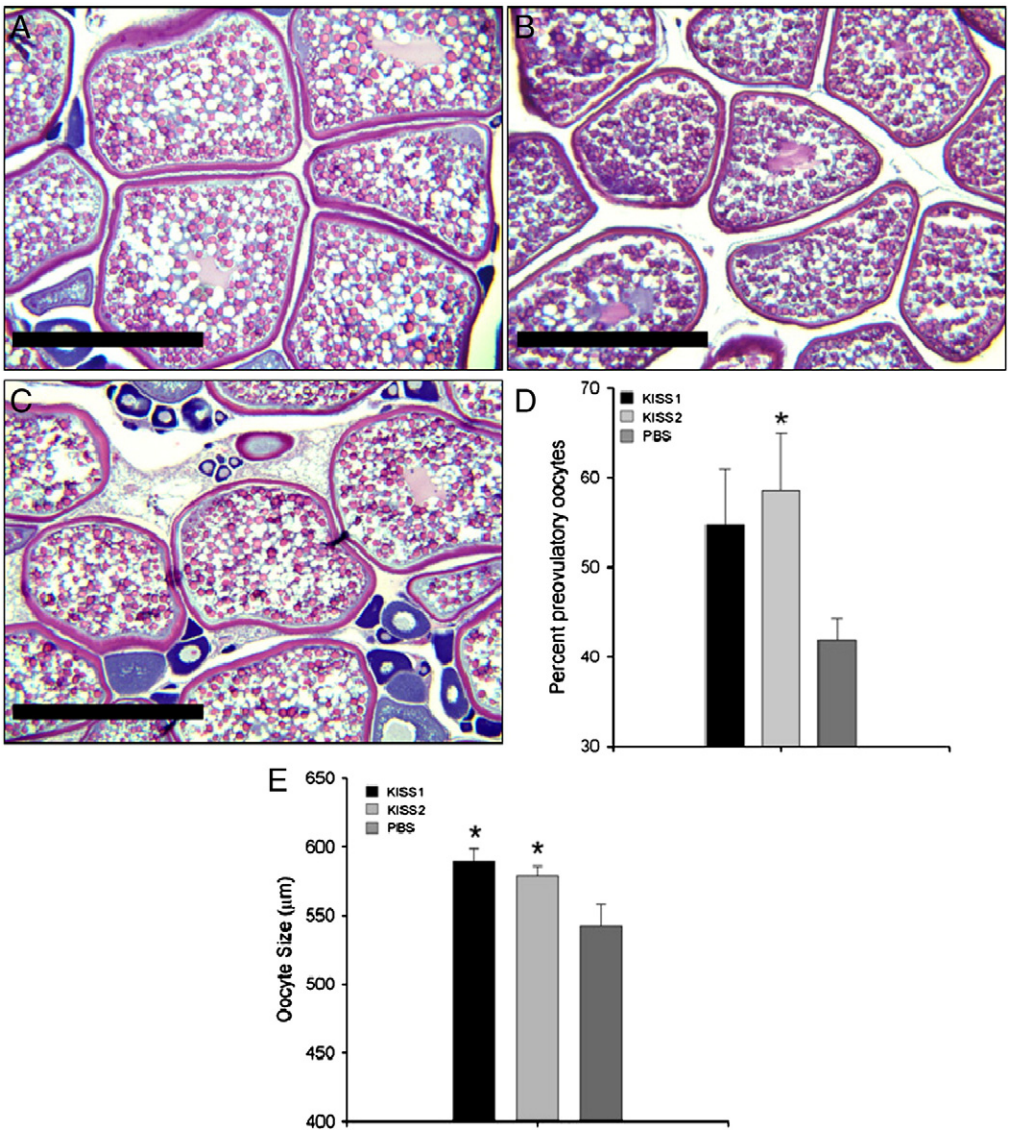

Fig. 6. Representative photomicrographs demonstrating ovarian development of adult white bass *Morone chrysops*. A.) KISS1 treated fish ovary showing maturing oocytes (stage VI) with abundant yolk granules and yolk vacuoles. Some primary follicles are present in the lower left near scale bar. B.) KISS2 treated fish with numerous maturing oocytes containing abundant yolk granules and yolk vacuoles. C.) PBS-treated fish with maturing oocytes containing abundant yolk granules and yolk vacuoles and numerous primary follicles (stages I–III). (Scale bar = 500 µm). D.) Percentage of preovulatory later stage (stage VI) oocytes present in histological sections by treatment. Asterisk indicates a significant difference from PBS injected fish.

suggesting that ovary and testes development could have been unstimulated or undetectably stimulated. Several reasons could account for this apparent lack of pubertal initiation in juvenile striped bass. First, the present study was not intended to exhaustively characterize the pharmacodynamics of kisspeptin treatments, thus, a more appropriate dose other than the one employed here may exist for juvenile striped bass. Second, it stands to reason that an alternative kisspeptin, if such exists, may induce stronger responses than the two kisspeptins utilized here. In fact, very recently it has been shown that striped bass possess copies of both KISS1 and KISS2 along with two forms of the kisspeptin receptor gpr54-1b and gpr54-2b (Zmora et al., 2011). In this study, a different kisspeptin sequence for striped bass was derived than the peptide sequences employed here. Another issue lies in the fact that only the kisspeptin decapeptide forms have been used for in vivo studies and different length forms may exert different potencies (Tena-Sempere et al., 2012). Moreover, the duration of treatment with our selected dose as well as the frequency of the injections (twice weekly) may have been too low to elicit an effect. Alternatively, the dose/duration of treatment may have been too great and resulted in a compensatory negative feedback as evidenced in a study of long term treatments of eel pituitary cells with various kisspeptins, where a consistent inhibition of lhβ was documented (Pasquier et al., 2011). Finally, there is evidence that in mammals the photoperiodic control of reproduction involves direct or indirect modulation of the kisspeptin system (Tena-Sempere et al., 2012). In our study, the species-specific photoperiodic cues may not have appropriately coincided with the treatment regimen. The lack of gonadal development in hybrid striped bass could further be explained by the unknown consequences of chromosomal recombination events through hybridization, as the appropriate molecular and hormonal signaling that kisspeptins may act upon could be dysfunctional or absent in hybrid striped bass. This possibility was not explored further in the current study and to our knowledge has yet to be explored in any hybrid species. Collectively, the lack of a response in both juvenile striped bass and hybrid striped bass is intriguing, and warrants further studies to better understand the KISS axis in these animals.

Interestingly, similar to the present study, both KISS1 and KISS2 have been shown to exhibit differential effects in various ages of fish.

In juvenile European sea bass (*D. labrax*), Felip et al. (2009) found that both KISS1 and KISS2 decapeptides induced some level of FSH and LH secretion within 1–2 h post-injection, but KISS2 exhibited superior effects. In adult sea bass, only KISS2 resulted in a modest increase in LH within a similar timeframe. In the present study, only KISS1 affected testes GSI in juvenile male white bass, but both decapeptides resulted in an increase in ovary GSI in juvenile female white bass; yet these same results were not recapitulated in adult white bass. The reasons for this differential age-associated efficacy are not clear, and future studies are needed to better elucidate kisspeptin effects within the context of fish age and gender.

A preponderance of the literature regarding reproductive aspects of Moronids focuses on the reproductive dysfunction of the parental striped bass; however, white bass sperm availability has been cited as a limiting factor in the generation of hybrid striped bass, because white bass commonly average less than 1 mL of milt at a single timepoint (Mylonas et al., 1997). The precise mechanistic cascade controlling the hydration or thinning of the milt in Moronids is not known. In white bass, androgen levels such as testosterone and 11-ketotestosterone spike in the latter part of spermatogenesis and spawning and may be associated with seminal hydration (Berlinsky et al., 1995). The injection of male broodstock multiple times throughout the spawning season with gonadotropic preparations, such as human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH) or their synthetic analogs has been employed to maintain hydration of the testis (Mylonas et al., 1997). This repeated handling can be very damaging, and might actually decrease sperm production due to increased stress (Bry et al., 1989). In most cases, these repeated gonadotropin injections lead to increased seminal fluid only, not increased spermatozoa (Clemens and Grant, 1965). No improvement in milt volume by the use of kisspeptins was observed here, but both decapeptide-treated groups did have higher spermatocrit values. These findings could suggest that greater volumes of spermatozoan-rich milt could be obtained if kisspeptins were used in concert with facilitators of hydration such as GnRH or hCG.

While closely related species, striped bass and white bass females employ different reproductive strategies. Unlike striped bass, which grow and ovulate only one clutch of oocytes per year, white bass have group synchronous oocyte development and are batch spawners (Berlinsky et al., 1995). In the current study, adult white bass females treated with either kisspeptin, but particularly KISS2, showed a weak trend toward increased GSI, had more oocytes at later stages of development, and both decapeptide treatments showed larger oocyte diameters. An increase in GSI, number of mature oocytes, and an increase in oocyte diameter as observed could represent a heightened synchrony whereby a larger pool of oocytes may have been available for the next ovulation event. Future efforts should focus on determining the nature of the relationship between kisspeptins and these gonadal metrics as well as the impact of such injections on current year and future fecundities.

Despite the small sample sizes of the large adult female striped bass encountered in the current study (due to enormous space requirements needed to house replicates of these fish), these individuals had already been through multiple annual reproductive cycles and demonstrated increased GSI in fish treated with KISS2. Like their congenerics, the white bass above, the importance of these findings is emphasized by the fact that the increases in GSI occurred without any temperature or photoperiod manipulation. While there were no differences in the follicular stages in any treatment of adult female striped bass, it stands to reason that the increase in GSI in KISS2-treated fish could have resulted in increased egg yields at spawning. However, no fecundity information was obtained from these individuals due to the desire to first examine ovarian development histologically. The increased weight gain by KISS1-treated in this group is intriguing, and warrants further investigation. In mammals, the KISS1 system has been closely linked to metabolic status and has been shown to be responsive to numerous metabolic signals associated with appetite regulation and energy intake and expenditure such as leptin, ghrelin, and neuropeptide Y (Pineda et al., 2010; Tena-Sempere et al., 2012). Recently, in Senegalese sole, *Solea senegalensis*, fasting was shown to increase KISS2 mRNA in the hypothalamus, which was associated with a concomitant rise in pituitary LH and FSH gene expression (Mechaly et al., 2011). Future efforts should focus on examining larger sample sizes of mature striped bass to determine the effects of kisspeptin treatments on actual fecundity and how kisspeptins may regulate metabolism in mature fish.

Detailed, long term research is clearly needed to comprehensively examine both associated signaling networks and feedback mechanisms as well as validate the very clear potential of these kisspeptins for basic scientists and commercial applications. Although it remains to be determined how kisspeptins may best be applied in commercial settings, our findings underscore the need for future studies to characterize the molecular underpinnings of the KISS system in various species, and in parallel, to design studies directly intended to approximate settings where the integration of kisspeptins into standard reproduction practices may result in dramatic improvements over current breeding paradigms.

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